ON THE SYNTHESIS AND FUNCTION OF THIAMINE PHOSPHATES

By

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ALMQVIST & WIKSELLS BOKTRYCKERI AB

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Preface

This paper is a summary of the following communications which will be referred to by the numbers given in the list of references at the end of this summary:

I. K.-H. Kiessling and P. E. Lindahl, Cocarboxylase as a Phosphate Carrier. *Arkiv Kemi 4* (1952) 285–287.

II. K.-H. Kiessling and G. Lindahl, Paper Chromatographic Separation of Thiamine and Its Phosphate Esters. *Arkiv Kemi 6* (1953) 271–272.

III. K.-H. Kiessling, Thiamine Triphosphate in Baker's Yeast. *Nature 172* (1953) 1187–1188.

IV. —— Studies on Thiamine Triphosphate. *Biochim et. Biophys. Acta 20* (1956) 293–298.

V. —— Incorporation of Radioactive Phosphate into Thiamine Phosphates in Yeast. *Acta Chem. Scand. 10* (1956) 831–839.

VI. —— Synthesis of Thiamine Diphosphate from Added Thiamine Monophosphate by Baker's Yeast. *Arkiv Kemi 10* (1956) 279–282.

VII. —— Paper Chromatographic Separation of Thiamine Phosphates. *Acta Chem. Scand. 10* (1956) 1356–1358.

VIII. —— Thiamine Phosphates and Phosphate Transport in Liver Mitochondria. *Acta Chem. Scand. 11* (1957) 917–926.

IX. —— Turnover Rate of Phosphate in Mitochondrial Thiamine Diphosphate. *Arkiv Kemi 11* (1957) 451–454.

X. —— The Effect of the Metabolization of Different Substrates upon the Incorporation of P^{32} into Mitochondrial Thiamine Diphosphate. *Arkiv Kemi 11* (1957) 469–470.

XI. —— The Incorporation of P^{32} into Mitochondrial Thiamine Diphosphate in the Presence of Different Substrates and Inhibitors. *Acta Chem. Scand. 11* (1957) 1062.

The investigations have been performed at the Institute of Zoophysiology, University of Uppsala, during the years 1949–1957, except for certain parts carried out in the Department of Biochemistry, University of Oxford, England, and the Wenner-Gren Institute for Experimental Biology, University of Stockholm.

I wish to express my sincere gratitude to Professor Per Eric Lindahl, who introduced me into this field of research, for steady encouragement and support, to Professor Hans A. Krebs for the privilege of carrying out part of the investigations in his Laboratory, and for the interest he has shown in it, and to Professor Olov Lindberg for valuable discussions and for all facilities put at my disposal when I had the favour of working in his Institute.

I am greatly indebted to Drs. Lars Ernster, Hans Löw, and Kerstin Lindahl-Kiessling, my wife, for indefatigable stimulation during the progress of this work.

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The English language has been revised by Professor Otto Zdansky and Dr. John Glomset.

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Introduction

Microbiological assay methods have made possible the discovery that thiamine (vitamin B_1) as well as the rest of the B vitamins are universally distributed in all living cells, whether of plant, animal, or bacterial origin.

Studies relating to the functioning of individual members of the B family of vitamins have demonstrated that they are integral parts of biological catalytic systems, and that they constitute essential factors in the metabolic machinery of widely diverse forms.

In this work the phosphate compounds of thiamine have been studied from different aspects: the isolation of synthetic and biological thiamine phosphates, their synthesis and their function as coenzymes in decarboxylation systems and in phosphate transport. The biological material has been liver from rat and guinea pig and baker's yeast. Depending on the striking resemblance exhibited in many respects in metabolic processes between the animal cell and the yeast cell, the study of a certain reaction in one of the cell types may often provide valuable information concerning the corresponding reaction in the other. The supply of material, the simplicity in treating it, and the technical aids available therefore become decisive factors when choosing material for a vitamin-enzyme investigation.

In certain respects yeast is preferable to animal tissues when studying the synthesis and function of the thiamine phosphates. The relative amounts of the thiamine compounds in yeast are much greater than in animal tissues, and the ability of further synthesis and accumulation exceeds greatly that of the liver, the organ richest in thiamine in the animal. Furthermore yeast is an easily available and not perishable material. The disadvantages are the difficulty of obtaining exactly reproducible results, especially if different kinds of yeast are used, but also with the same type.

The methods used in isolating and investigating compounds which occur only in very small quantities present great difficulties. At the beginning of this work no micromethods for the isolation of thiamine phosphates had been elaborated. In the search for such a method the first problem was to find a suitable paper chromatographic solvent. In the course of the investigations six different paper chromatographic separation methods have been used. On this account, part of a special chapter of the present summary has been devoted to methods of this kind.

In the course of my studies on thiamine phosphates in biological material I have never succeeded to prove the occurrence of thiamine triphosphate in animal tissues. It was, however, possible to demonstrate its presence in small quantities in yeast. Moreover, a higher concentration could easily be obtained by suitable treatment of the cells. This is the reason why yeast has been used when investigating the role of biological thiamine triphosphate. For the study of its participation in the phosphate transport in animal tissues the synthetic compound was used.

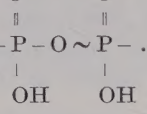
On the degradation of a substrate, for example pyruvate, to carbon dioxide and water by means of a series of transformations involving oxidations and decarboxyla-

tions, the energy, especially liberated in the oxidation steps, is partly bound in the form of energy-rich bonds. The bonds said to be of biological importance are those with phosphorus and sulphur and with a value of free energy of hydrolysis ($-\Delta F_0$) of about 10,000 cal. The phosphorus may be bound in different ways, as in

acetylphosphate: $-C(=O)-O\sim P(OH)_2 \cdot O$,

phosphocreatine: $=N\sim P(OH)_2 \cdot O$,

or adenosine triphosphate:



With regard to the arrangement of the phosphate groups the thiamine phosphates are comparable with the adenosine phosphates (26). Thus thiamine diphosphate should involve one energy-rich bond, and thiamine triphosphate two.

As thiamine diphosphate is known to act as a coenzyme in oxidative decarboxylations of α-ketoacids, i.e., in reactions where the requirements for formation of energy-rich bonds exist, this compound, as well as other thiamine phosphates, have been studied with special attention to the turnover of their phosphate groups.

The part of the present work which deals with the phosphate turnover in thiamine phosphates in animal tissues was started in 1948 simultaneously with the publication by Green *et al.* of the work about the cyclophorase system. This made it possible to work with an isolated, organized enzyme system containing the entire complement of enzymes and coenzymes necessary for the total oxidation of pyruvate and all the intermediates of the Krebs cycle to carbon dioxide and water.

In 1950 the methods of Hogeboom and Schneider for isolating mitochondria widened the possibilities of investigating these organized reactions, but gave at the same time rise to lively discussion concerning the question of whether the cyclophorase complex was identical with the mitochondrion or if it had to be regarded as a system which, compared with the mitochondrion, exhibited in certain respects a disturbed organization. Nowadays the latter opinion prevails, and consequently my investigations of thiamine phosphates and phosphate transport, done on the cyclophorase complex, were once more taken up and extended to mitochondria prepared according to Hogeboom and Schneider.

With radioactive inorganic phosphate in a biological system the labelling of a thiamine phosphate, initially present in the system or added as a synthetic compound, may be brought about in different ways. One possibility is the formation of thiamine phosphates by synthesis. Synthesis here means that radioactive phosphate groups (from adenosine phosphates) are transferred to the thiamine compound with an increase of the amount of the phosphorylated compound as a result.

As another possibility the thiamine phosphate already formed, either free or protein bound, might incorporate P^{32}, either by exchange of its phosphate with other phosphate compounds, the phenomenon being independent of reactions consuming or producing energy (Fig. 1, Alt. I), or as an intermediate in a phosphate transporting

chain coupled to the generation of energy-rich bonds (Fig. 1, Alt. II). In the figure X ~ P symbolizes a thiamine phosphate containing at least one energy-rich phosphate bond, A ~ P, B ~ P, C ~ P, and D ~ P energy-rich phosphate compounds not containing thiamine.

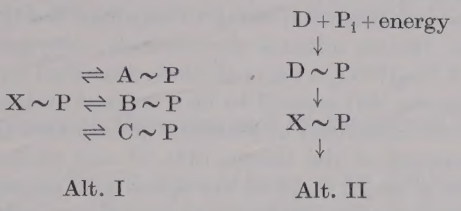


Fig. 1.

When in the separate papers and in the present summary thiamine phosphates are considered with reference to phosphate transport or phosphate metabolism the alternatives depicted in Fig. 1 are referred to in contrast to synthesis.

I. Isolation and Chemical Synthesis of Thiamine Phosphates

In the course of his classical researches on fermentation Neuberg (45, 46) in 1911 discovered the enzyme carboxylase, which converts pyruvic acid into acetaldehyde, and in general α-keto carboxylic acids into the corresponding lower aldehydes by decarboxylation. Whereas in the earlier stages of the work on this important component of the enzymatic fermentation complex it was held that in contrast to the zymase–cozymase system no coenzyme was required by carboxylase, Auhagen (1), in 1932, was able to demonstrate cocarboxylase as an essential constituent of the carboxylase system. The attempts to isolate cocarboxylase led to preparations containing only small amounts of active substance.

Five years later Lohmann and Schuster isolated the compound in pure form from yeast (42). They started from 100 kilograms of brewer's yeast, and obtained 0.7–0.8 gram crystalline substance (about 15 per cent of the original amount present in the yeast), the composition of which was equivalent to thiamine diphosphate (TDP).[1]

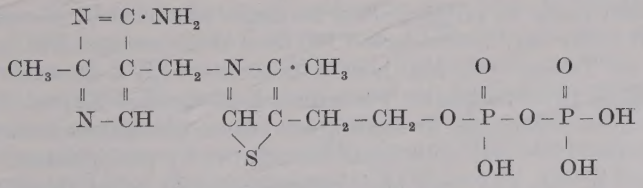


Fig. 2. Thiamine diphosphate (cocarboxylase).

On account of the low yield their methods for isolating thiamine phosphates are, however, not suitable for small amounts of tissue. The methods published by Consden,

[1] Terms and abbreviations: T, thiamine (vitamin B_1); TMP, thiamine monophosphate; TDP, thiamine diphosphate (cocarboxylase); TTP, thiamine triphosphate; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; P^{32}, radioactive phosphate; P_i, inorganic phosphate.

Gordon, and Martin 1944 (7) for the paper chromatographic separation of amino acids opened a new approach to the isolation of small quantities of thiamine compounds.

Primary chromatographic experiments were performed on synthetic mixtures of thiamine phosphates, my intention being to separate the thiamine compounds from inorganic phosphate, flavine adenine dinucleotide, cozymase, and adenosine phosphates. A mixture of Na_2HPO_4 – amyl alcohol, described by Carter (6) and modified by Snellman and Gelotte (58) seemed to be a solvent suitable for this purpose. This solvent served also for separation of thiamine phosphates from tissue extracts after appropriate pre-treatment of the tissues (32). It was exchanged later for a solvent described by Burrows *et al.* (5) in which the spots were concentrated to smaller areas. With these methods, however, different thiamine phosphates were not separated from each other.

During the years 1950–54 several paper chromatographic methods for separating different thiamine phosphates from each other were reported (59, 70, 33, 3, 63). Only with three of these (33, 3, 63) is TTP separated from TDP, and on account of the very low R_f value of the compounds in all solvents the separations are not satisfactory, especially for TTP and TDP from biological extracts. Besides the position of the TTP spot always coincides with that of nucleotides, so that phosphate analyses on TTP can not be done without careful removal of the interfering components before the chromatographic separation.

My intention was to find a solvent in which TMP, TDP, TTP, and thiamine phosphates with more than three phosphate molecules were separated from each other. In addition TTP and TDP should simultaneously be separated from nucleotide di- and triphosphates. Among the many solvents examined the most suitable was found to be one used among others by Krebs and Hems (35) for separating nucleotides, and which is a modification of a method described by Zetterström and Ljunggren (82). With this solvent ten synthetic thiamine compounds could be separated from each other. Two of them were different forms of thiamine, followed in turns by thiamine mono-, di-, tri-, tetraphosphate and four additional thiamine phosphates with more than four phosphate groups (23). The R_f value of every nucleotide di- and triphosphate was lower than those of TTP and TDP, permitting thereby the removal of the nucleotides.

In order to facilitate the study of the biological function of thiamine phosphates efforts have been made to prepare them in larger amounts by chemical synthesis. 1937 Stern and Hofer (64) synthesized TDP from thiamine and $POCl_3$. This method was modified by Tauber (67) who heated thiamine with a mixture of phosphoric acid and inorganic pyrophosphate. Viscontini, Bonetti, and Karrer (71) prepared a mixture of thiamine phosphates by heating anhydrous phosphoric acid with thiamine. From this mixture TDP and TTP could be isolated by precipitation, but the compounds obtained in this way were still contaminated by other thiamine phosphates as was shown by means of paper chromatography (57). Only after separation with paper chromatography (30, 23), or column electrophoresis (30, 54) pure compounds could be obtained.

Also thiamine phosphates labelled with radioactive phosphorus have been prepared. By means of the methods of Tauber (67) and of Viscontini *et al.* (71), Ogata *et al.* (49) obtained TDP equally labelled in the two phosphate groups. In my experiments I needed thiamine phosphates labelled only in their hydrolyzable phosphate. This was brought about by heating an anhydrous mixture of phosphoric acid and radio-

active inorganic phosphate with TDP for 15 minutes at 100°C (27). The thiamine phosphates formed were precipitated according to Viscontini *et al.* (71) and separated by paper chromatography (23). The synthesis could be carried out with either TMP or TDP, but TMP is less easily procurable. The thiamine phosphates synthesized in this way contain about 99 per cent of their radioactivity in the hydrolyzable phosphate.

II. Occurrence of Thiamine and Its Phosphate Esters

Thiamine occurs in many different types of living cells. Williams and Spies (80) list in tabular form about ninety different types of sources of thiamine. These include mammalian tissues, fish, dairy products, legumes, cereals, vegetables, fruits, nuts, and yeast. Investigations carried out in the laboratories of Williams show that thiamine (as well as the rest of the B vitamin family) are to be found in any type of biological material examined.

A disulfide form of thiamine was first prepared by Zima and Williams (83), and found to be biologically equivalent to thiamine. Later when Fujiwara *et al.* (14) discovered the disulfide allithiamine, a new interest in this type of substance arose. A team under T. Matsukava have reported their investigations about thiamine derivatives in a series of publications. Marked variations as to the occurrence of different thiamine disulfides in different plant species were found (for references see *Annual Review of Biochemistry* (1955) 419).

Following upon the work of Auhagen (1) and Lohmann and Schuster (42) it has been realized that a considerable part of the thiamine in yeast and in animal tissues is combined with phosphate to TDP which may undergo cleavage resulting in free thiamine.

According to Sperber and Renwall (62) baker's yeast contains only ten per cent of thiamine in its free form, the rest being TDP. In animal tissues, as for instance liver, brain, and kidney, a very large percentage of the total thiamine is present as TDP, while in muscles the concentration of free thiamine may nearly equal (or in some cases exceed) that of TDP (48, 77). Plants often contain very little of their thiamine in the form of TDP, but many unexplained irregularities occur.

Actually the TDP itself partly occurs in protein-bound form in the holoenzyme, the carboxylase. Carboxylase has been isolated in pure form by Green, Herbert, and Subrahmanyan (17), and contains 0.46 per cent TDP and 0.13 per cent magnesium.

The distribution of TDP between different fractions of rat liver cells has been studied by Goethart (15). The mitochondrial and soluble fractions prepared by centrifugation contained practically equal proportions of the cellular TDP. The nuclear fraction and the microsomes were essentially free of TDP. By means of ultrafiltration Goethart showed that the cytoplasmatic TDP mainly occurs as free TDP, i.e., not bound to protein (16).

By paper chromatography Rossi-Fanelli, Siliprandi, and Fasella (52) isolated from an extract of rat liver thiamine, TMP, and TDP and in addition a compound which they identified as thiamine triphosphate (TTP). The concentration was about two thirds of that of TDP and about the same as TMP. Using a paper chromatographic method, employed in our first investigation (32) of TDP in liver, we found a very weak spot with an R_f value somewhat higher than that of TDP. It was thought to be TTP, but as the separation method was very unsatisfactory, we did not report our

findings. Later, when more suitable paper chromatographic methods were available, it was impossible to reproduce our earlier observation. From yeast, however, to which had been added an excess of thiamine three hours before the killing of the yeast cells, chromatograms were obtained showing a very distinct spot which corresponded to chemically synthesized TTP (22). In these experiments a new solvent was used (31).

Also in yeast not pre-incubated with thiamine small amounts of TTP could be found (24). The occurrence of TTP in yeast was recently confirmed by Ziro by means of ion exchange chromatography (85).

III. Synthesis of Thiamine Phosphates in Yeast and Animal Tissues

Lohmann and Schuster reported in 1937 (42) that TDP could be synthesized from thiamine in biological material, more exactly in slices of pigeon brain and in intestine bray. ATP, creatine phosphate, and phosphopyruvate accelerated the synthesis. The same year Euler and Vestin (11) succeeded in obtaining the synthesis of TDP from thiamine in dried brewer's yeast, in the presence of ATP or hexose diphosphate. Stern and Hofer (63) tried, without success, to isolate an enzyme from rat liver and brain which phosphorylated thiamine. In consequence of investigations done on a phosphatase-free aceton powder from pig duodenal mucosa Tauber (66) maintained that the enzyme phosphorylating thiamine to TDP[1] was not the same as that which dephosphorylated TDP. Ochoa (47) and Sperber (60), however, arrived at the opposite conclusion, while Westenbrink *et al.* (78) gave further support to Tauber's opinion.

Lipton and Elvehjem (40, 41) studied the phosphorylation of thiamine in brewer's yeast, and proposed the following reaction for the phosphorylation:

$$ATP + T = AMP + TDP$$

that is, thiamine is phosphorylated by directly receiving a pyrophosphate group from ATP.

Weil-Malherbe held the same mechanism of phosphorylation to exist in a partly isolated enzyme system from brewer's yeast (72, 73). This system phosphorylated thiamine to TDP in the presence of ATP and, after the formation or addition of TDP, pyruvate could be decarboxylated by the same system. Without added thiamine compounds no decarboxylation occurred. TDP was formed more rapidly from thiamine than from TMP, and from this Weil-Malherbe concluded that TMP could not be directly phosphorylated to TDP, but was primarily dephosphorylated to thiamine. Finally, by receiving a pyrophosphate group from ATP, it was transformed into TDP.

This argumentation presumes the presence of an acid phosphatase affecting thiamine phosphates. This, according to Westenbrink *et al.* (76), has its optimum at pH 4. The occurrence of such a phosphatase in the enzyme system isolated by Weil-Malherbe has been questioned (65) for the following reason: on addition of thiamine together with TDP to his protein fraction, containing apocarboxylase and thiamino

[1] This enzyme has been called thiamino kinase by Steyn-Parvé (65), and this term will also be used by me in the following.

kinase, a stimulating effect on the carboxylase action should be expected, the stimulation being due to inhibition of the TDP destruction by the phosphatase (74, 76). Nevertheless, Weil-Malherbe did not observe any such effect of thiamine.

Steyn-Parvé (65) isolated a thiamino kinase reportedly free from phosphatases from baker's yeast. With this enzyme TDP was formed from both thiamine and TMP. The rate of the synthesis from TMP was about half of that from thiamine, as was also the case with the enzyme system used by Weil-Malherbe.

Thoai and Chevillard (68) isolated a thiamino kinase from brewer's yeast, phosphorylating both thiamine and TMP to TDP. ATP and ADP were about equally active as phosphate donors, irrespective whether thiamine or TMP were started from. The system did not contain adenylate kinase. As distinguished from the thiamino kinase isolated by Steyn-Parvé, which precipitates between 50 and 60 per cent saturation with ammonium sulphate, this precipitates between 45 and 50 per cent.

From the soluble fraction of cell protein (fraction not sedimented at 25,000 *g*) Leuthardt and Nielsen (39), using rat liver, isolated an enzyme which catalyzed the formation of TDP from either thiamine or TMP in the presence of ATP. TMP yielded only one fourth as much TDP as did thiamine. Hence they conclude that TMP is hydrolyzed to thiamine, and then receives a pyrophosphate group from ATP.

A recent publication by Forsander deals with the phosphorylation of thiamine (12). He isolated a thiamino kinase from baker's yeast, and by extraction with phosphate buffer, precipitation with $(NH_4)_2SO_4$, heat precipitation, reprecipitation with $(NH_4)_2SO_4$, adsorption on $Ca_3(PO_4)_2$ gel, and finally again precipitation with $(NH_4)_2SO_4$ he obtained a fairly pure enzyme with good activity. By using ATP labelled only in the terminal phosphate group he showed that with this enzyme thiamine is phosphorylated to TDP by a direct transfer of a pyrophosphate molecule according to the reaction below:

$$T + AP^{31}P^{31}P^{32} = TP^{31}P^{32} + AP^{31}.$$

It would thus seem unquestionable that thiamine can be phosphorylated by the transfer of a pyrophosphate group from ATP. The results of Thoai and Chevillard on the other hand presented evidence that also another way exists. The contradictory results may depend on the existence of two different thiamino kinases in baker's and brewer's yeast which in the systems isolated from the two types of yeasts might be present in different amounts. Thus when TMP gives rise to TDP with the enzyme from baker's yeast as is the case to a certain extent in the investigations of Steyn-Parvé (65) and Weil-Malherbe (72, 73), this may be due to the presence of a monophosphorylating enzyme as well as of a pyrophosphorylating enzyme. The former enzyme has been entirely removed by the isolation process of Forsander (12). Consequently, Thoai and Chevillard (68) might have obtained mainly the monophosphorylating enzyme.

It is possible also that baker's yeast contains only a pyrophosphorylating thiamino kinase, and that the synthesis of TDP from TMP, observed by Steyn-Parvé, was due to the presence of phosphatases in her enzyme preparation. This possibility has been suggested by Forsander (12), but seems less probable in view of the demonstration by Steyn-Parvé that no hydrolysis of thiamine phosphates occurs during an incubation. It might further be objected to the experiments of Steyn-Parvé that nothing is reported about the thiamine content of the TMP used by her. Even a slight contami-

nation with thiamine might yield TDP in such amounts that it could be interpreted as being synthesized directly from TMP.

In order to find out if baker's yeast can synthesize TDP from TMP without prior hydrolysis to thiamine, I have attempted to use a yeast system which deviated as little as possible from the intact yeast (25). Thiamine is taken up by intact yeast cells without difficulty (62, 84), whereas the cells probably are impermeable to thiamine phosphates (25). If therefore thiamine phosphates are added to a yeast suspension they are hardly able to reach the thiamino kinase on account of difficulties in penetrating the cell membrane. Thus the cells had to be made permeable to TMP. This was done by freezing-thawing the yeast repeatedly before incubation (25).

Another difficulty to account for is that phosphatases very probably are situated on the surface of the yeast cells. When TMP is added the compound should thus rapidly be hydrolyzed to thiamine and free phosphate.

This opinion has been advanced by Sperber (61), but is not in agreement with the findings of Westenbrink *et al.* (78). However, the following experiment, which I have not had the opportunity of publishing earlier, strongly supports Sperber's opinion:

Fresh baker's yeast was incubated in a phosphate buffer with TMP free from thiamine, glucose, and magnesium. Samples were taken at different intervals and analyzed for the occurrence of free thiamine in the medium after the yeast cells had been removed by centrifugation. The amount of free thiamine in the medium rapidly rose during the first ten minutes after which it decreased. Paper chromatograms showed that after ten minutes nearly all TMP was hydrolyzed to thiamine. The formation of thiamine from TMP very likely was due to hydrolysis of the TMP before penetration into the cells. The drop of the amount of thiamine in the medium after ten minutes depended on the fact that the formation of thiamine from TMP no longer covered the decrease through active uptake into the cells.

To this it may be objected that no definite proof is given of the permeability of the cell wall to TMP. Thus TMP might have penetrated the cell wall, and might subsequently have been hydrolyzed by phosphatases within the cell, and then, as free thiamine, passed out of the cell again. But as it has been shown that thiamine is rapidly and to a large extent taken up by the yeast by means of an active transport mechanism (62, 84), it seems very improbable that thiamine, once inside the cell membrane of the intact yeast cell, should pass out into the medium, and then once more enter the cell.

Molybdate was found partly to inhibit the phosphatases. After two hours' incubation of TMP together with yeast, in the presence of molybdate, seventy per cent of the TMP still remained (25). Accordingly, the experiments, intended to clarify the synthesis of TDP, were carried out in the following manner: TMP, radioactive inorganic phosphate and molybdate were added to yeast cells pre-treated by freezing-thawing. After two hours' incubation the TDP synthesized was isolated. It was found that the TDP exhibited a higher radioactivity in the βP than in the αP (P^{32} of αP was about sixty per cent of the P^{32} of βP). From this I concluded that baker's yeast is able to synthesize TDP from TMP without previous hydrolysis of the TMP to thiamine.

Theoretically $\alpha P^{32}/\beta P^{32}$ should be almost zero in yeast treated by freezing-thawing. No complete inhibition of the phosphatases could, however, be obtained as pointed out earlier, and the amounts of thiamine formed from TMP by still active phosphatases very likely suffice for the saturation of the pyrophosphorylating system mentioned

before (12). Further addition of thiamine, as in the experiment with thiamine + TMP (25), did not change the ratio $\alpha P^{32}/\beta P^{32}$, confirming the hypothesis that already in the experiment with TMP alone the amount of thiamine formed is sufficient for the enzyme system starting the synthesis from this compound.

Consequently two systems exist, one which phosphorylates thiamine directly to TDP (12) by means of transferring a pyrophosphate group from ATP, and another having the ability to phosphorylate at least TMP to TDP by monophosphorylation.

In baker's yeast incubated with an excess of thiamine it is possible to show, in addition to TMP and TDP, also the occurrence of TTP (22, 26). The ability of yeast to synthesize the latter compound may be regarded as an additional argument in favour of the existence of a monophosphorylating thiamino kinase. Yet, an enzyme of the same type as adenylate kinase might give rise to TTP from TDP. However, preliminary experiments (unpublished results) did not support this view.

In this connection it may be of interest to note that different kinds of baker's yeast synthesize their TTP under different conditions. Only two examples shall be referred to here. For the Swedish yeast (from Uppsala Ångkvarn, Uppsala, Sweden) the optimal conditions were the same as those given for the synthesis of TDP by Sperber and Renwall (62) and by Westenbrink *et al.* (78) with glucose as a substrate in succinate buffer 0.123 *M*, pH 3.7 (22). However, an English baker's yeast (from the United Yeast Company, Ltd., Manchester), while yielding a satisfactory synthesis of TDP under identical conditions gave only very small amounts of TTP. Much more TTP was synthesized when pyrophosphate buffer, pH 6.5, was used (26).

IV. Reactions Catalyzed by Thiamine Coenzymes

A. *Thiamine, Thiamine Monophosphate, Thiamine Diphosphate*

Whether thiamine plays a physiological role or is only stored in the tissues is uncertain. It has been shown that in yeast the free thiamine activates the carboxylase (48, 76), but that in animal tissues the carboxylase is not influenced (74). The effect very likely depends on the inhibition of phosphatases.

Woolley and Merrifield (81) presented evidence for a metabolic function of thiamine not mediated through TDP, but did not discuss in detail the possible reaction in which thiamine might take part.

TMP is generally considered not to have any coenzyme function.

Enzymatic reactions in which TDP has been demonstrated to function as a coenzyme are listed in Table 1.

Another reaction in which TDP is supposed to take part is the decarboxylation of succinate to propionate (79) and the carboxylation of propionate to succinate shown recently by Lardy and Adler to occur in mitochondria (36), but the role of TDP is still obscure.

Zima and Williams (83) and later Myrbäck, Vallin, and Magnell (44) proposed a mechanism where thiamine should occur alternately in oxidized and reduced condition. This was postulated on the basis of the observed thiamine activity of thiamine disulphide pyrophosphate, a dimer in which the thiazole nucleus can open. Then a possibility should exist for TDP to take part in the carrier system. However, this point of view has been discarded, and instead the -SH is supposed to react with pyruvate (20). Mitzuhara and Handler (43) have proposed an essentially similar

TABLE 1. *Reactions catalyzed by enzymes containing TDP as coenzyme*

Quoted after Forsander (12)

Reaction	Enzyme
Pyruvic acid → acetaldehyde + CO_2	carboxylase
α-ketobutyric acid → CO_2 + propionaldehyde	carboxylase
Benzaldehyde + pyruvic acid → acetylphenylcarbinol	carboligase
2 pyruvic acid → acetoin + 2 CO_2	carboligase
2 diacetyl + H_2O → acetoin + 2 acetic acid	diacetylmutas
Benzoyl formic acid → CO_2 + benzaldehyde	benzoylformiate-decarboxylase
Pyruvic acid + CoA + DPN → acetyl-CoA + + $DPNH_2$ + CO_2	pyruvic acid oxidase
α-ketoglutaric acid + CoA + DPN → succinyl--CoA + $DPNH_2$ + CO_2	α-ketoglutaric-dehydrogenase
Ribulose-5-phosphate + ribose-5-phosphate → 3-phosphoglycerinealdehyde + sedoheptulose-7-phosphate	transketolase

formulation. They suggest, however, that the tertiary nitrogen of TDP rather than the S of the thiol form should coordinate with pyruvate to form an intermediate which is decarboxylated to an "active acetaldehyde" carrying molecule. The only difference between the two hypotheses is whether the N or the S carries the aldehyde group. In either case the reaction can be visualized as a decarboxylation to yield an active aldehyde which may then react in various ways, depending on the organism or enzyme system with the following results: acyl lipoate as in the pyruvate system of *Escherichia coli* (21); acetaldehyde in the *Acetobacter suboxydans* system, where the decarboxylation reaction precedes the oxidation (33); acetoin by condensation; by oxidation acetyl TDP or acetate or in the usual mammalian system to acetylate lipoic acid either free or as a part of the same molecule (lipothiamine) (51).

B. *Thiamine Triphosphate*

Identification of thiamine triphosphate from yeast

TTP has been shown to occur in liver (52) and in yeast (22, 26, 85). With the paper chromatographic separation method used in my first investigation on TTP (22) the R_f value obtained for the actual thiamine compound from yeast was the same as for synthetic TTP, and therefore the yeast compound was assumed to be a triphosphate of thiamine. However, in this solvent the compounds moved only a very short distance. With another solvent (26) the R_f values were higher (yeast compound 0.64, synthetic TTP 0.66). This difference in R_f values was supposed to depend on a possible impurity still present in the yeast TTP (26). However, in the course of later experiments the mean value of about fifty chromatograms revealed no real difference between the R_f value of the two compounds. Slight variations in both R_f values could be obtained by varying the quantities in the spots, and this may explain the differing figures.

Phosphorus analyses of the biological thiamine compound, behaving as synthetic TTP on the paper chromatograms, showed that three molecules of phosphorus were

coupled to each thiamine, and for this reason the name thiamine triphosphate could be maintained.

My intention was to find out if the yeast TTP was identical with synthetic TTP with respect to the arrangement of the three phosphate groups. The groups could either be arranged in the same way as in ATP, that is, bound to each other by pyrophosphate linkages, or only two groups could be in a pyrophosphate linkage and the third bound as a monophosphate group on another place of the thiamine molecule. Polyphosphate esters of this type have been synthesized by Roux, Teysséire, and Duchesne (55). In order to settle this problem, acid hydrolysis of biological TTP was carried out as well as enzymatic hydrolysis with potato apyrase. With acid two phosphates were split off after a ten minutes hydrolysis, and the remaining monophosphate compound was found to have the same R_f value as thiamine monophosphate. Synthetic TTP behaved identically after acid hydrolysis.

Potato apyrase prepared according to Lee and Eiler (38) has been shown only to hydrolyze γP from ATP at 0°C and both γP and βP at 30–40°C. Incubating synthetic TTP and yeast TTP with potato apyrase gave at 0°C mainly TDP in either case, and at 40°C TMP (26). The results from the paper chromatographic analyses were confirmed by phosphorus determinations.

Substitution of TDP by synthetic TTP

The ability of synthetic TTP to replace TDP as a coenzyme in the pyruvate decarboxylating system has been investigated by Velluz *et al.* (69), Siliprandi and Siliprandi (57), de la Fuente and Diaz Cadavieco (13), Rossi-Fanelli *et al.* (52, 54), and Kiessling (26). In all investigations carboxylase from yeast has been used. The results vary greatly. Velluz *et al.* found that synthetic TTP could replace TDP in a washed yeast system, but four times more TTP than TDP was required. Their TTP had not been purified by chromatography. With chromatographically isolated synthetic TTP Siliprandi (57) found TTP to be as active as TDP. De la Fuente and Diaz Cadavieco using synthetic TTP purified according to Siliprandi and a yeast apocarboxylase isolated according to Green *et al.* (18) found TTP to be inactive. Their results have recently been verified by Rossi-Fanelli (54) and Kiessling (26) who also showed that this apocarboxylase did not contain phosphatases. The earlier results may depend on the contamination of TTP with TDP, as was probably the case in the investigations of Velluz *et al.* (69), or on the presence in the crude yeast apocarboxylase of sufficient phosphatase activity to split active TDP from TTP, producing the appearance of TTP being as active as TDP. This was the interpretation given by Siliprandi (57). These questions have been discussed among others by Rossi-Fanelli *et al.* (54) and Kiessling (26).

An entirely different explanation of the contradictory results has recently been put forward by Bartos (4) who suggests the existence, in yeast, of two distinct apoenzymes, one combining with TDP, the other with TTP.

Substitution of TDP by biological TTP

TTP isolated from yeast and purified as described above (26) was added to purified yeast carboxylase with pyruvate as a substrate. The system showed no decarboxylation activity. Only when the TTP had first been treated with potato apyrase at 0°C decarboxylation did occur (26). This was also the case when synthetic TTP was used

instead of yeast TTP. The carboxylase preparation did not contain any phosphatases. This shows that TTP isolated from yeast (as well as synthetic TTP) cannot act as coenzyme in a pyruvate decarboxylating system isolated from yeast. Only when pre-treated with an enzyme which splits off γP and thus transforms it into TDP is decarboxylation obtained. The negative results can not depend on contamination of the TTP with inhibiting components as TDP obtained from TTP by enzymatic hydrolysis is very active (26).

Synthetic TTP has been studied in a similar way by Velluz *et al.* (69). Incubating TTP with apyrase at 37°C, and taking samples at different intervals in order to register decarboxylation activity in a washed yeast system they found, that initially the activity increased in order to decrease after longer incubation intervals.

The results of phosphate analyses, paper chromatographic comparisons, enzymatic hydrolyses, and studies of the decarboxylation activity referred to above, indicate no differences, chemical or physiological, between synthetic TTP and TTP isolated from yeast.

V. Thiamine Phosphates and Phosphate Transport

A. *Animal Tissues in the Absence of Synthetic Thiamine Phosphates*

When starting our investigation about the incorporation of P^{32} into thiamine phosphates we thought the most suitable system would be the cyclophorase system described by Green *et al.* (19). The system is an integral fraction of particles obtained by washing tissues homogenized with a Waring blender in isotonic salt solution, and contains the entire complement of enzymes and coenzymes necessary for the total oxidation of pyruvate and all the intermediates of the Krebs cycle to carbon dioxide and water. The enzyme system is named cyclophorase system in order to emphasize that it is more than a simple mixture of enzymes and coenzymes.

To this enzyme complex we added radioactive inorganic phosphate and pyruvate and, as "sparking components", fumarate and bicarbonate. The incubation was performed for twenty minutes under oxygen (32). TDP was then isolated, and the specific radioactivity of its phosphorus determined. The same incubation was carried out without "sparking components". Each incubation contained enzyme from twelve grams of guinea-pig liver. In contrast to subsequent experiments no synthetic TDP was present during the incubation nor added during the isolation. The large amounts of enzyme containing TDP made nevertheless the isolation of the compound possible.

From the experiments it was evident that the radioactivity of TDP was higher in the pyruvate metabolizing system compared with that where the omission of bicarbonate and fumarate had resulted in very slow metabolism. A certain proportionality existed between the amount of oxygen consumed and the incorporation extent of P^{32} into TDP.

While this work was being carried out a lively discussion was going on as to whether fundamental differences existed between the cyclophorase system and the enzyme system which formed the intact mitochondrion. Gradually the opinion crystallized that the cyclophorase complex originated from the mitochondrion, and that the mitochondrion from a physiological point of view was to be considered a more intact

enzyme system, due mainly to the technique used when isolating the two systems. For example, a high ATPase activity was found in the cyclophorase complex which did not occur in the mitochondria prepared in sucrose. An intense accumulation of inorganic pyrophosphate was likewise typical for the cylophorase complex (34, 8). Also the organization in the mitochondrial membrane is affected in the preparations of Green, and gives rise to a different permeability to added substrates, compared with mitochondria prepared in sucrose (50). The enzyme systems which have been shown to be localized in the membrane (56) may also possess divergent properties. In consequence of differences of these types, which may be of importance in connection with the phosphate turnover, the investigation of the participation of thiamine phosphates in the phosphate transport had to be extended also to include mitochondria prepared in sucrose. The reactions would in this case proceed under conditions where the shape and physiological properties of the mitochondria were preserved to the greatest possible extent.

Thus mitochondria prepared from guinea-pig liver were incubated with radioactive inorganic phosphate and with pyruvate as a substrate (27). In order to maintain a high oxidative phosphorylation during the incubation, hexokinase and glucose were added in excess according to Lardy *et al.* (37). In this way energy-rich phosphate is trapped off from ATP to glucose, and the amount of ADP, functioning as receiver of energy-rich phosphate continuously formed, is high and constant during the experiment. When after 20 minutes' incubation TDP and ATP-ADP were isolated, and specific radioactivity determined, the radioactivity of the mitochondrial TDP was shown to be $\frac{1}{2}$–$\frac{2}{3}$ of that of ATP-ADP. Mainly the terminal phosphate group was radioactive.

In another incubation with the same additions as those described above, but without added substrate, the incorporation of P^{32} into TDP was considerably lower. In proportion to the oxygen consumption it was, however, of such a magnitude that no straight correlation was obtained between oxygen consumption and incorporation into TDP as had been the case with the cyclophorase system.

The results with mitochondria prepared in sucrose, referred to above, derive from three types of experiments slightly differing from each other in some respects. In the first type trichloroacetic acid was added to the suspension directly after the incubation was finished, in the second the mitochondria were again centrifuged down, and washed with sucrose before the addition of the acid. In this case much of the inorganic P^{32} and other additions were removed already at the beginning of the isolation, but here the fragility of the mitochondria increasing with their being kept too long even in sucrose carries with it the risk of losses of mitochondrial TDP (9). In a third type different amounts of mitochondria were incubated with the same amount of additions. In all three cases TDP incorporated P^{32} in accordance with the figures above, and the incorporation, as was shown in the third type, was proportional to the amount of mitochondria.

The labelling of TDP can be explained either by synthesis of TDP from thiamine (see page 6), or by Alternative I or II, Fig. 1. As has already been mentioned in an earlier chapter most of the thiamine of the liver is present in the form of TDP and only a small fraction as free thiamine. But since free thiamine actually occurs in the tissues the possibility exists that the radioactivity of the isolated TDP is simply due to synthesis of TDP from thiamine.

Against this theory the following objections can be raised: if TDP were synthesized from thiamine both phosphate groups should be about equally labelled. In my experi-

ments mainly the βP of TDP became radioactive. Furthermore, Leuthardt and Nielsen found that the synthesis of TDP in liver is mainly confined to the cytoplasm (39).

Experiments in which the incorporation of P^{32} into the cytoplasmic TDP was compared with that into mitochondrial TDP gave additional support to the hypothesis that the labelling was not exclusively the result of a mere synthesis. Supernatant obtained during the isolation of mitochondria from 15 grams of liver was incubated in the same way as had been done with the mitochondria, and the radioactivity of the cytoplasmic TDP was compared with that of TDP in mitochondria obtained from the same amount of liver (27). Both incubations were performed with pyruvate as a substrate. The radioactivity of TDP from the cytoplasm rose to only $\frac{1}{5}$ of that of the mitochondrial TDP, and was slightly lower than that in mitochondria without substrate.

NaF in a final concentration of 0.04 *M* was found by Ochoa (47) only slightly to inhibit the synthesis of TDP from thiamine in animal tissues. When adding NaF to mitochondria (28) I found a complete inhibition of the incorporation of P^{32} into TDP already with concentrations which lay fifty per cent below those used by Ochoa. This may be taken as a further evidence for the incorporation being not only a result of a mere synthesis of TDP from thiamine present in the mitochondria.

The rate of the incorporation of P^{32} into TDP with pyruvate as a substrate has been compared with that into ATP-ADP (29). Samples were taken at different intervals. After seven minutes the radioactivity of the hydrolyzable P of mitochondrial TDP was about forty per cent of that of ATP-ADP. After this point the specific radioactivity of ATP-ADP did not increase further as it had reached that of inorganic phosphate. The amount of P^{32} in TDP still rose, but along a gentler curve.

The influence of different substrates (pyruvate, α-ketoglutarate, succinate, and fumarate) upon the incorporation of P^{32} into mitochondrial TDP has been studied (30, 28). With pyruvate and succinate a stronger labelling was obtained than with α-ketoglutarate and fumarate. In the last cases the labelling of TDP was only slightly higher than without any substrate at all. It was surprising that the metabolization of succinate favoured the incorporation of P^{32} into TDP more than α-ketoglutarate did, since TDP is known especially to take part in the decarboxylation of α-ketoacids. Admittedly succinate may give rise to the α-ketoacid pyruvate in mitochondria, the oxidative decarboxylation of which caused a marked incorporation of P^{32} into TDP. This can, however, not explain the incorporation with succinate as a substrate since fumarate, an intermediate formed when succinate gives rise to pyruvate, did not cause incorporation of P^{32} into TDP to the same extent as succinate. This conclusion was further supported by results obtained with amytal (28). According to Ernster *et al.* (10) amytal (5-ethyl-5-isoamylbarbiturate) completely inhibits hydrogen transfer from mitochondrial dehydrogenases to oxygen in DPN-linked oxidation systems. Since succinate oxidation is not coupled to DPN, amytal does not influence its aerobic oxidation or the phosphorylations coupled to it. But other steps, as for instance the oxidation of malate or pyruvate, are inhibited.

In my experiments with succinate as a substrate the presence of amytal only slightly inhibited the incorporation of P^{32} into TDP (28). This means that the incorporation is not caused by oxidative decarboxylation of pyruvate formed from succinate, but that it depends either on the transformation of succinate to fumarate by succinic dehydrogenase or, possibly, on another reaction involving succinate. Succinic dehydrogenase does not contain TDP, whereas an enzyme system, found

by Lardy and Adler (36) to occur in liver mitochondria and which decarboxylates succinate to propionate, needs TDP. Unfortunately this decarboxylation proceeds very slowly, at least in their enzyme preparation (mitochondrial extract), and detracts from this possibility of explaining the incorporation into TDP with succinate as a substrate. Further investigation of the problem is, however, in progress.

As mentioned in the introduction (page 6) the labelling of the intramitochondrial TDP in the experiments described above may be explained in different ways. I have tried to demonstrate that synthesis is not the main reaction. It is, however, hard to say which of the two alternative reactions in Fig. 2 is involved. The oxidation of α-ketoglutarate or succinate or pyruvate ought to result in the formation of about equal amounts of energy-rich phosphate bonds (A ~ P, B ~ P, C ~ P), the oxidation of the last two substrates nevertheless causing a higher incorporation of P^{32} into TDP than the first one. This does not support the first alternative but instead strengthens the possibility that TDP may be a link in a phosphate transferring chain (Alternative II, Fig. 1), intimately coupled to the degradation of pyruvate and succinate.

The experiment with DNP added to the mitochondria, where DNP showed no inhibiting effect on the incorporation, makes, however, Alternative II less acceptable (about the inhibiting action of DNP see O. Lindberg and L. Ernster, *Protoplasmatologia III* (1954) p. 67).

B. *Animal Tissues in the Presence of Synthetic Thiamine Phosphates*

The above investigations have all been performed on mitochondrial TDP, that is, no added TDP has been present during the incubations.

Bartley 1954 published results from experiments with a kidney cyclophorase system in which he studied the incorporation of P^{32} into thiamine phosphates (3). He worked with small amounts of enzyme (2 ml of the "R_3 residue") to which he added, before incubation, relatively large amounts of synthetic TDP (final concentration 0.005 *M*). After various intervals he isolated TDP by means of paper chromatography. Pyruvate, α-ketoglutarate, and succinate were tried as substrates. In all cases the incorporation of P^{32} into TDP was low. However, pyruvate and succinate gave higher values than α-ketoglutarate. No permeability barriers for TDP existed.

Bartley interpreted the low incorporation rate of P^{32} into TDP (with pyruvate the radioactivity of TDP after 20 minutes was only 5 per cent of that of ATP, and with α-ketoglutarate 1 per cent) as proving that TDP does not, to any appreciable extent, act as a phosphate carrier. From his results this is obviously the case with added TDP, whereas no conclusions can be drawn about the radioactivity of the TDP originally bound in the enzyme at the beginning of the experiment.

My experiments performed on mitochondria prepared in sucrose show that, at least with this enzyme system, synthetic and mitochondrial TDP incorporate P^{32} in a different degree (27). The specific radioactivity of synthetic TDP was less than 1 per cent of that of mitochondrial TDP.

As in the case of the cyclophorase system no permeability barriers existed for the added thiamine phosphates in the mitochondria. This was shown by incubating mitochondria with synthetic thiamine phosphates, isolating the mitochondria after incubation, washing them twice and, after removal of the proteins by precipitation,

adding the neutralized extract on a paper chromatogram (unpublished experiments). The amount of TDP was about the same in the mitochondrial fraction as in the surrounding medium.

Bartley showed that the amount of added TDP decreases continuously during the incubation with the cyclophorase complex. Whether this depends on the presence of phosphatases or on the direct transfer of the phosphate of the TDP to another compound, for example ADP, is not clear. Ogata *et al.* (49) on the other hand have shown by means of labelled TDP, that in a cyclophorase system a direct transfer of phosphate occurs from TDP to the adenosine phosphates, especially if pyruvate or α-ketoglutarate are oxidized. With succinate as a substrate this effect is less evident. The reaction is inhibited by NaF, but not by DNP.

In order to find out if the same reaction occurs in a mitochondrial system I have synthesized thiamine di-, tri-, and tetraphosphate labelled only in their hyrolyzable phosphate groups, and added them together with ADP, glucose, and hexokinase to mitochondria prepared in sucrose (27). If a direct transfer of phosphate from the added thiamine phosphates to ADP takes place, hydrolyzable radioactive phosphate should be transformed into non-hydrolyzable glucose-6-phosphate by means of hexokinase.

The results, however, show that in this type of mitochondrial system no, or in any case a very slow direct transfer of phosphate occurs from added thiamine phosphates to ADP.

The contradictory results might possibly be explained by the assumption that the cyclophorase complex is to be regarded in certain respects as partially destroyed mitochondria. In comparison with the intact mitochondrion the actual transphosphorylating enzyme in the cyclophorase system should be much more accessible to added thiamine phosphates. Once having delivered their phosphate, the thiamine compounds would again be released from the enzyme giving place to another thiamine phosphate molecule. No remarkable rephosphorylation of the thiamine compound would occur, either because the system was not working under strict physiological conditions, or because thiamine phosphates were present in excess. That with TDP in excess only a very slow rephosphorylation occurs, is obvious from the results of Ogata *et al.*, which show, that on addition of non-labelled TDP together with radioactive inorganic phosphate the incorporation of P^{32} into ATP is very much slower than after addition of labelled TDP.

C. *Baker's Yeast*

The relative amount of thiamine phosphates, as was pointed out earlier, is higher in yeast than in animal tissues, and can be considerably increased above normal by the addition of thiamine (62, 78, 22, 26). This is not the case with animal tissues (47). The uptake of thiamine in yeast is an active process (62, 84), and the compound is transformed in the yeast cells mainly to TDP, but also to TMP and TTP (22, 26). The ratio between TTP, TDP, and TMP after pre-incubating English baker's yeast (from the United Yeast Company, Ltd., Manchester) with an excess of thiamine was found to be 22:100:13 (26). Very likely the relatively high amount of TMP is due not exclusively to hydrolysis of TDP and TTP during the isolation, since after exposing synthetic TDP and TTP to the same isolation steps, employed with the biological compounds, I have found only a very slight hydrolysis. It has furthermore

been shown that TMP, in a certain degree, may exist as an intermediate in the synthesis of TDP in yeast (25). About the further significance of TMP nothing is known.

The role of TTP in the yeast cells still remains unexplained. When studying the incorporation of P^{32} into thiamine phosphates in baker's yeast a divergent result was, however, obtained for TTP as compared with TDP and TMP (24). The experiments included on the one hand yeast containing much thiamine phosphates, brought about by pre-incubation with an excess of thiamine, on the other hand yeast which had not been pre-treated in this way. In the second type of experiments the amounts of thiamine phosphates were so small that no reliable quantitative figures could be obtained. It was, however, evident that P^{32} was incorporated into TDP and TMP at a very slow rate, while after the addition of glucose TTP became rapidly labelled. The labelling was mainly localized to the terminal phosphate.

On the whole these results are confirmed by experiments with yeast made rich in thiamine phosphates (24). Here too the γP of TTP was more radioactive than the α- and βP and also more than the phosphate groups of TDP and TMP. As thiamine, to a certain extent, exists in the unphosphorylated state in the yeast (62) and was present in excess before incubation in the pre-incubated yeast the question arises: is the labelling of TTP caused only by incorporation of P^{32} during synthesis of TTP from thiamine or by an exchange of the phosphate in the already existing TTP. However, most of the thiamine was washed out of the pre-incubated cells before the addition of radioactive inorganic phosphate (established by paper chromatography). Furthermore neither TMP nor TDP, at least one of them very possibly being an intermediate in the synthesis of TTP, were labelled to the same degree as the γP of TTP. These two facts do not support the theory about labelling by synthesis. A more likely explanation of the high turnover rate of radioactivity in the terminal phosphate of TTP, when glucose is metabolized, might be found in the assumption that TTP takes part in metabolic phosphate transport (Alternative II, Fig. 1) or interchanges phosphate with phosphate containing compounds rapidly labelled in the presence of a substrate (Alternative I, Fig. 1).

Summary

The investigations include studies on synthetic and biological thiamine phosphates, their synthesis, isolation, function as coenzymes in decarboxylation reactions, and participation in metabolic phosphate transport.

1. Paper chromatography proved to be very suitable for the separation of different thiamine phosphates from each other and from other phosphate containing compounds. From synthetic mixtures of thiamine phosphates pure samples of thiamine mono-, di-, tri-, and tetraphopsphate, respectively, could be obtained. The pure compounds were then used for investigating their participation in biological reactions.

2. In addition to the prevailing view that the synthesis of thiamine diphosphate in yeast occurs as a result of the direct transfer of a pyrophosphate group from ATP to thiamine, it has been shown that a monophosphorylation of thiamine monophosphate can occur apparently parallel to the former reaction.

3. Besides thiamine mono- and diphosphate a thiamine phosphate, not previously known to occur in yeast, has been isolated. By means of phosphate analysis, enzymic

phosphate hydrolysis, and paper chromatography this was proved to be thiamine triphosphate. The addition of an excess of thiamine to the yeast greatly increased the concentration of the compound.

4. Thiamine triphosphate from yeast, as well as synthetic thiamine triphosphate did not exhibit cocarboxylase activity when tested in an isolated pyruvate decarboxylating system. Activity was obtained only after pre-treatment with potato apyrase under conditions which led to the splitting off of only one phosphate group.

5. Incubation of baker's yeast with radioactive inorganic phosphate without addition of a substrate gave only rise to a slight incorporation of P^{32} into the thiamine phosphates. The addition of glucose resulted in a rapid renewal in thiamine triphosphate of its phosphate and, mainly, the terminal group.

6. Intramitochondrial thiamine diphosphate was shown to incorporate radioactive phosphate, when mitochondria (from liver) were incubated with a substrate as pyruvate or succinate in the presence of radioactive inorganic phosphate, but less with α-ketoglutarate, fumarate or without added substrate. The effect upon the incorporation of inhibitors as DNP, NaF, and amytal were studied. Different mechanisms for the incorporation are discussed.

Synthetic thiamine phosphates added to the mitochondria did not incorporate P^{32} in the same degree as the intramitochondrial thiamine diphosphate.

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